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13. ABSTRACT (Maximum 200 Words)
The successful treatment of breast cancer requires detection of the disease at early stages. In this application, we propose to systematically isolate secreted and cell surface proteins (trafficked proteins) with increased expression in early stage breast tumors. These proteins are candidates for blood-borne markers and cell surface markers that can be used in the routine screening of early stage breast cancer. We proposed to first isolate all secreted and cell surface proteins from breast tumors of multiple patients, using a functional approach we have designed and validated. Next, the expression levels of these proteins in normal and early stage breast tumor tissues will be compared, and those with increased expression in tumors will be identified and analyzed. To date, a cDNA library has been constructed using human breast tumor samples. This library has been subjected to genetic screens to enrich cDNA fragments that encode signal peptides for trafficked protein. The library enriched for trafficked protein has been validated in preliminary analysis. DNA sequencing analysis is underway to further confirm the quality of the enriched library. Micro-array analysis will be carried out to identify the trafficked proteins with increased expression in human breast tumor tissues.

14. SUBJECT TERMS
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Introduction

The successful treatment of breast cancer requires detection of the disease at early stages. Currently the diagnostic tools for breast cancer suffer from drawbacks. For example, mammography can miss small lesions, and sometimes can induce tumors in certain patients. Tests of biopsied tumor samples can only be performed in symptomatic patients when the tumor has been identified. Certain blood-borne markers (such as PSA) have been used in affordable, routine serum tests to diagnose cancer in asympotamatic patients. However, these markers, when used individually, can detect the presence of only a certain percentage, but not all, of a particular type of tumors. This argues that a panel of blood-borne markers should be identified and used for the diagnosis of each type of cancer. It is conceivable that multiple blood-borne markers, when used in combination, could predict the existence of tumors more accurately and more successfully. Once the presence of a tumor is predicted, cell surface markers can be used in imaging analysis to identify the location of the lesion.

In this application, we propose to systematically isolate secreted and cell surface proteins (trafficked proteins) with increased expression in early stage breast tumors. These proteins are candidates for blood-borne markers and cell surface markers that can be used in the routine screening of early stage breast cancer. We will first isolate all secreted and cell surface proteins from breast tumors of multiple patients, using a functional approach we have designed and validated. Next, the expression levels of these proteins in normal and early stage breast tumor tissues will be compared, and those with increased expression in tumors will be identified and analyzed. These studies will generate a pool of potential biomarkers, which can be evaluated through further studies for their use in the early diagnosis of breast cancer.

Body

We constructed a secretion trap library with $1.5X10^7$ independent clones from 15 human breast tumor samples. Two secretion trap screens were performed using this library to isolate cDNA fragments encoding the signal sequences of the trafficked proteins in human breast tumor tissues.

Screen #1.

In our first screen, we packaged the library as a whole into retroviruses by transfecting the library DNA into a retroviral packaging cell line, LinX-A. 100 10-cm plates of 293 cells, each containing $2X10^6$ cells, were infected with the library viruses. A parallel infection with a control virus expressing the LacZ gene indicated an infection efficiency of 20-30%. Therefore, we estimated that at least $4X10^7$ cells had been infected, which was sufficient to cover the complexity of the library with a more than 2-fold redundancy. After purification of infected cells by hygromycin treatment, cells were subjected to selection with magnetic beads coupled to an anti-CD8 antibody. Because the yield of the first round of selection was expected to be low due to the low percentage of CD8 positive cells in the initial population, we decided to perform the selection in two

large pools in order to shorten the period of recovery before the cells were ready for the second round. 10^7 cells were detached from each plate with 3 mM EDTA/EGTA and combined into 2 pools, with each pool containing $5X10^8$ cells from 50 plates. Each pool was selected with 2.5 ml of Dynabead M-450 CD8 in 50 ml of phosphate-buffered saline (PBS) containing 5% fetal calf serum and 0.6% sodium citrate in 50 ml Falcon tubes. After washing with the same buffer, the bound cells were eluted with 500 µl of DETACHaBEAD, and plated into four 10-cm plates. The subsequent rounds of selection was performed in Eppendorf tubes with 50 µl of Dynabead M-450 CD8 in a total volume of 1 ml, and the bound cells were eluted with 10 µl of DETACHaBEAD. After each selection cycle, a portion of the recovered cells was immuno-stained with an anti-CD8 antibody coupled to a florescent dye (FITC), and the percentage of CD8 positive cells was determined by flow cytometry. After four rounds of selection with CD8 beads, the CD8 positive cells were enriched to about 80% for both pools.

The integrated proviruses were excised by treating the genomic DNA isolated from selected cells with Cre recombinase, and recovered after electroporation into E. coli. 20 single colonies were picked, and the plasmid DNA was isolated and sequenced to determine the identity of the cDNA fragment in each clone. Unfortunately, we found that both pools were dominated by 2-3 cDNA species. This finding indicated that this secretion trap screen failed due to the overgrowth of a few founder cells that had acquired growth advantage. Thus, the lesson we have learned from our first screen is that cells transduced with the library need to be kept as separate as possible during selection and growth.

Screen #2.

The strategy for our second secretion trap screen was designed to keep the cells transduced with the library in as many pools as possible during the selection process. In addition, we divided the library into pools of $2X10^5$ before packaged into viruses, in order to reduce the possibility that the growth-promoting cDNAs are present in all pools of the cells transduced with the library. The glycerol stock of the library was divided into 96 pools, each containing $2X10^5$ bacterial cells. Plasmids were isolated from these pools using a Qiagen robot. These pools of DNA were transfected separately into LinX-A packaging cells, and the resulting virus pools were used to infect 293 cells in 96 10-cm plates, each containing $2X10^6$ cells. At an infection efficiency of 20-30% (as determined in a parallel infection with a control virus expressing the LacZ gene), we estimated that each pool of the library had been transduced into $4X10^5$ cells, and that the total number of cells transduced by the library was $3.84X10^7$. After purification of transduced cells with hygromycin, 10^7 of cells from each pool were selected separately with 50 µl of anti-CD8 beads in a total volume of 1 ml in an eppendorf tube, and the bound cells were eluted with 10 µl of DETACHaBEAD. Eluted cells from each set of 4 plates were combined and plated into a fresh 10-cm plate for recovery. Therefore, after the first round of selection, the number of pools was reduced to 24, which were renamed from A to X. These 24 pools were kept separate during the following rounds of selection, which again was performed in Eppendorf tubes with 50 µl of Dynabead M-450 CD8 in a total volume of 1 ml.

After each round of selection, a portion of the recovered cells was stained with an anti-CD8 antibody coupled to FITC, and the percentage of CD8 positive cells were determined by flow cytometry. After 4 rounds of selection, most of the pools contained more than 40% of CD8 positive cells, with the exception of pools S and V (Table 1 and Fig. 1A). Three pools, H, K and O, were contaminated at various stages during selection, and were excluded from further analysis. As controls, we also performed parallel selection with 293 cells transduced with either pTRAP alone or a combinaiton of pTRAP containing a CD8 signal sequence (pTRAP-SS) and pTRAP mixed at a ratio of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} or 10^{-6}. As expected, the CD8 positive cells were gradually enriched in the control cell populations transduced even with the lowest percentage (10^{-6}) of pTRAP-SS, but not in that transduced with the pTRAP vector only (Table 2 and Fig. 1B). This results indicated that the selection was successful.

The proviruses have been recovered from 19 pools (A-G, I, J, L-N, P-R, T, U, W and X) in which CD8 positive cells had been enriched to more than 40%. Restriction analysis of randomly selected clones from each pool indicated that most of these pools were represented by multiple cDNA species of different sizes, with the exception of 4 pools that seemed to be dominated by 2-3 different types of cDNA. Therefore, it seems that our new strategy has solved the problem we encountered in our first screen, and that the domination of a small number of clones did not occur in the majority of the pools in the second screen. We are currently performing further analysis of these pools by DNA sequencing. Once we have confirmed that the signal sequences are enriched in these recovered pools, they will be subjected to micro-array analysis.

POOL #	Pre-Selection	First Round	Second Round	Third Round	Fourth Round
A	0.2	1.8	5.2	24.1	57.3
B	0.1	1.2	2	20	60.7
C	0.1	1.5	6.7	43.3	68
D	0.1	0.9	1.1	12.3	47.5
E	0.1	1.1	2.9	21.9	68.6
F	0.2	2.6	16.7	73.8	87.2
G	0.6	2.5	10.4	44.3	75.6
I	0.1	0.6	6.8	44.3	70.2
J	0.2	1	10.2	43.3	68.6
L	0.2	2.7	12.3	44.6	63.5
M	0.2	0.9	1.5	21	49.2
N	0.1	0.8	1.4	13.5	49.8
P	0.2	2	9	48.3	62.5
Q	0.2	0.8	7.2	34.3	61.8
R	0.2	1.2	4.6	19.1	40.1
S	0.1	0.7	6.1	23.1	39
T	0.1	0.5	5.5	34	48.4
U	0.1	1.1	14.5	27.5	44.2
V	0	0.6	1.3	2.7	9.5
W	0.3	3.8	40.1	66.2	67.1
X	0.2	0.9	13.2	40.3	63.7

Table 1. % of CD8 positive cells after each round of selection with magnetic beads coupled to an anti-CD8 antibody. The initial cell populations were transduced with pools of the secretion trap library from human breast tumors. After each round of selection, a portion of the cells was stained with an anti-CD8 antibody coupled to FITC and analyzed by flow cytometry.

	Pre-Selection	First Round	Second Round	Third Round	Fourth Round
0	0.1	0.4	0.3	0.4	0.5
10^{-2}	10.6	46.1	62	73.6	81.5
10^{-3}	0.3	4.1	45.4	76.5	78.5
10^{-4}	1	9.9	33.4	55.4	70.2
10^{-5}	0.4	1.4	16.5	33.2	51.8
10^{-6}	0.1	0.3	1.4	9.2	25.8

Table 2. % of CD8 positive cells after each round of selection with magnetic beads coupled to an anti-CD8 antibody. The initial cell populations were transduced with a mixture of the pTRAP vector containing a CD8 signal sequence and the pTRAP vector mixed at a ratio of 0, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. After selection, part of the cells was stained with an anti-CD8 antibody coupled to FITC and analyzed by flow cytometry.

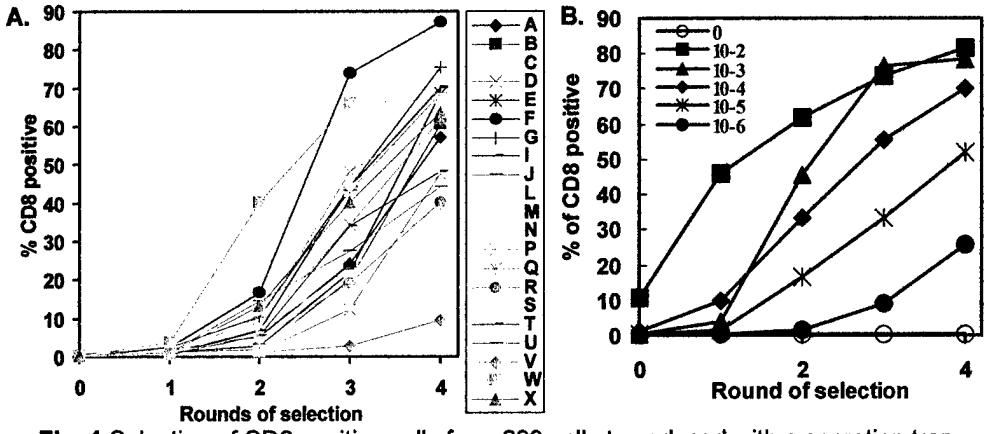

Fig. 1 Selection of CD8 positive cells from 293 cells transduced with a secretion trap library derived from human breast tumors or control plasmids. % of CD8 positive cells after each round of selection with magnetic beads coupled to an anti-CD8 antibody. Cells were transduced with pools of a secretion trap library from human breast tumors (A) or with the pTRAP vector containing a CD8 signal sequence and the pTRAP vector mixed at a ratio of 0, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.

Key research accomplishments

We have obtained a human breast tumor library in which the signal sequences have been enriched.

Reportable outcomes

We have obtained a human breast tumor library in which the signal sequences have been enriched.

Conclusions

We have obtained a human breast tumor library in which the signal sequences have been enriched. The micro-array analysis will be carried out after further validation of this library by DNA sequencing.

References None.

Appendices None.